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Susceptibility of Steelhead Trout 'Salmo gairdneri' Richardson to Redmouth Infection 'Yersinia ruckeri' Following Exposure to Copper

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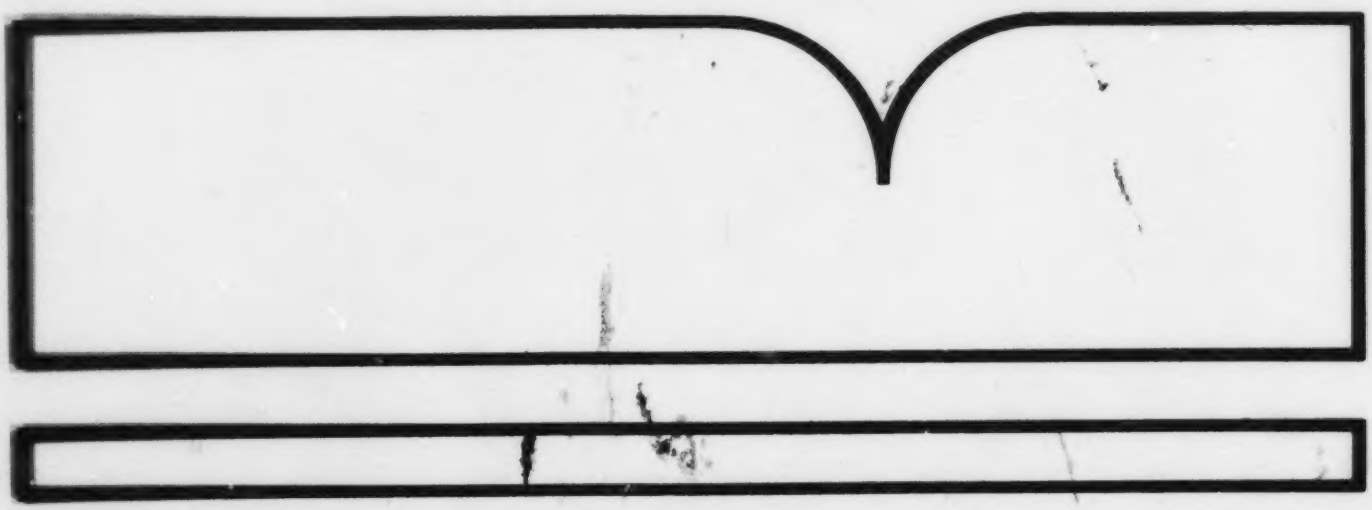

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JOURNAL ARTICLE

Susceptibility of steelhead trout *Salmo gairdneri* Richardson to redmouth infection *Yersinia ruckeri* following exposure to copper

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Abstract. Steelhead trout were exposed to sublethal concentrations of copper and inoculated with *Yersinia ruckeri*. Copper exposure at 7 and 10 μg/l for 96 h caused more fish to die of infection than control fish (no copper). Infection susceptibility increased with time of exposure to a single dosage of copper (10 μg/l), reaching a maximum at 48 h. Lowering the copper concentration to 5 μg/l caused the infection susceptibility to occur at 24 h. The infectious dose of *Y. ruckeri* was lower in fish exposed to 10 μg/l copper for 48 h than control fish.

Introduction

Heavy metals, in particular copper, are known to be toxic to fish. For example, Chapman (1978) reported that the 96 h copper LC_{50} for various life stages of steelhead trout *Salmo gairdneri* Richardson is in the range of 17–38 μg/l. It has been recognized that environmental changes can cause a higher prevalence of disease in natural populations of fish (Brown, Sinclair, Keith, Beamer, Hazdra, Nair & Callaghan 1977; Brown, Koch, Sinclair, Spitzer & Hazdra 1979) and are contributing factors in the development of stress in fish (Wedemeyer 1970). Rodsaether, Olafsen, Raa, Myhre & Steen (1977) presented evidence that eels, *Anguilla anguilla* L. latently infected with *Vibrio anguillarum*, died of vibriosis after being exposed to copper-containing water. This link between exposure to sublethal copper concentrations and disease was further demonstrated by Hetrick, Knittel & Fryer (1979). They exposed rainbow trout to copper as low as 3·9 μg/l and found the fish were more susceptible to infectious haematopoietic necrosis (IHN) virus infection than fish exposed to the virus only.

This paper presents results that illustrate copper-exposed steelhead trout are more susceptible to *Yersinia ruckeri* infection (enteric redmouth bacterium, ERM) than control fish.

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Materials and methods

Fish

Fish used in these experiments were obtained from a local state hatchery as 'eyed' eggs. When brought to the laboratory they were sanitized for 10 min in a 1:250 (v/v) solution of Wescodyne (West Chemical Co., N.Y.)* buffered to pH 7·0 with $NaHCO_3$. This procedure minimized the possibility of disease transfer from the hatchery to our laboratory. The fish were reared in our laboratory on a diet of Oregon moist pellet, and when used in the experiments they had a mean weight of $8{\cdot}0 \pm 4{\cdot}1$ g and a mean length of $8{\cdot}7 \pm 1{\cdot}4$ cm.

Exposure of fish to copper

Fish were continuously exposed to copper in a proportional flow-through toxicant dilutor developed by Garton (1980). A stock solution of copper was prepared with reagent grade $CuCl_3.2HO$. The copper was stabilized by addition of 0·1 ml of concentrated nitric acid per litre. The acid did not appreciably change the pH of the dilution water, usually less than 0·1 pH unit between the high and low copper concentrations. The dilutions of copper were delivered at 1·5 l/min to glass aquaria ($86{\cdot}7 \times 22{\cdot}5 \times 39{\cdot}5$ cm) holding 58 l of water. Ten aquaria received the copper-containing water which provided five concentrations, in duplicate, ranging from <1 to 10 μg/l. Two additional aquaria received ambient water only and served as a non-copper control. Water temperature was maintained at 12°C during copper exposure. Photoperiod was maintained and adjusted periodically to correspond with the local day length.

Twenty-five fish were placed in each aquarium and allowed to acclimatize for 48–96 h before the copper flow was begun. They were exposed up to 96 h and then removed for infection. A total of 50 fish (25 in each of two aquaria) was exposed at each copper concentration, from which 25 fish were removed for infection. The remaining fish were held as copper stress controls. Twenty-five fish were also removed from the ambient water aquaria for infection. This latter group served as a control for the level of *Y. ruckeri* infection in the absence of copper stress.

The handling stress between copper exposure and infection was eliminated in later experiments (all experiments after the effect of copper concentration) by constructing a dilutor (Garton 1980) for the aquaria used in the infection phase. This dilutor provided single or proportional dilutions of copper to a 48-l aquaria. Copper flow to any aquarium could be stopped and the fish infected without handling.

Duplicate 10-ml samples of water were taken daily during copper exposure from each aquarium for copper determination. The samples were acidified with 0·01 ml of concentrated nitric acid and the copper concentration was determined with a Perkin-

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Elmer atomic absorption spectrophotometer (Model 305B) equipped with a flameless HGA-2000 heated graphite atomizer. The copper levels employed ranged from <1 to 10 μg/l depending on the experimental design. All these levels are in the sublethal range for steelhead trout (Chapman 1978).

Water chemistry

Since the effective copper concentration is influenced by water hardness, daily samples were taken for analysis of water hardness and alkalinity. The data are reported in the graphs and tables. The chemical makeup of the well water used in these experiments has been reported by Samuelson (1976) and Chapman (1978).

Bacterium

A strain of *Y. ruckeri*, isolated and identified from an epizootic, was maintained in the laboratory at 5°C on trypticase-soy-agar (TSA) (Difco, Detroit, Michigan) slants. Cultures were transferred to fresh medium once a month.

Infection of fish

Cultures of *Y. ruckeri* were grown in 200 ml of sterile trypticase-soy-broth (TSB) at 25°C for 24 h. This culture was diluted with sterile TSB to an optical density of 0·6 at 660 nm using a spectrophotometer. Water-flow was turned off in the aquarium and 200 ml of the diluted culture were added. This was an inoculum of 1×10^3 bacteria per ml. Contact was continued for 1 h and the water flow was resumed (1 l/min).

Necropsy

Mortalities were removed daily from each aquarium. The surface of each fish was sanitized by immersion in a 10% (v/v) solution of Wescodyne for 5 min. An incision was made aseptically to expose the kidney. Samples of the kidney tissue were removed with a sterile inoculation loop and transferred to TSA plates. The plates were incubated at room temperature for 48 h. Typical *Y. ruckeri* colonies were removed and confirmed by inoculating API-E strips (Analytab Plainview, N.Y.) and by mixing with anti-*Y. ruckeri* serum (Department of Microbiology, Oregon State University, Corvallis, Oregon). Only those mortalities which yielded cultures that were culturally and serologically identical to *Y. ruckeri* were counted as being caused by *Y. ruckeri* infection.

Results

Effect of copper concentration

When steelhead trout were exposed to a range of copper concentrations from ambient

to 10 μg/l it was found that fish were stressed at the high copper concentration. The results (Table 1) show that mortality due to infection was greater in those fish exposed to copper concentrations of 7·0 and 10·6 μg/l than fish receiving the bacterium only. These data were found to be significant at $\alpha = 0{\cdot}05$ by the *t*-test. The fish exposed to copper levels of 2·7 and 4·0 μg/l showed no greater susceptibility to infection than those exposed to the bacterium only. No mortalities occurred in fish exposed to copper only, showing that increased mortality at 10·6 and 7·0 μg/l copper was not due to the residual effect of copper.

Table 1. Effect of copper concentration on the susceptibility of steelhead trout to infection by *Yersinia ruckeri* 96-h exposure

Mean copper concentration (μg/l)		Mean water hardness as $CaCO_3$ (mg/l)	Fraction dying	Fraction positive *Y. ruckeri*	Percentage mortality
10·6		63·5 ± 0·5	14/25	14/14	56*
7·0		63·5 ± 0·5	10/25	10/10	40*
4·0		63·5 ± 0·5	6/25	6/6	24
2·7		63·5 ± 0·5	6/25	6/6	24
0·0	(control-bacteria only)	63·5 ± 0·5	5/25	5/5	20
10·6	(control-copper only)	63·5 ± 0·5	0/25	0/0	0

* Significant at $\alpha = 0{\cdot}05$ by *t*-test.

Effect of time exposure

The time required for copper-exposed fish to show an increase in infection susceptibility is shown in Table 2. Copper-exposed fish were more susceptible to infection after 24 h compared to the non-exposed fish. Copper exposure for 48 h further increased susceptibility. By 96 h of copper exposure, the fish showed a decline in susceptibility to a level similar to those exposed for 24 h. These data were found to be significant at $\alpha = 0{\cdot}05$ by the *t*-test. Again, no mortalities occurred among those fish exposed to copper only.

Acclimatization to copper

The preceding experiment suggested that infection susceptibility due to copper exposure did not continue to increase with time but reached a peak. This experiment was designed to determine if the fish could overcome the effects of copper and the susceptibility to infection decline (Table 3). Those fish exposed to the copper (5·7 μg/l) for 1 day had the greatest infection susceptibility. Fish exposed to copper for

Table 2. Time to maximum infection susceptibility to *Yersinia ruckeri* during exposure of steelhead trout to a single concentration of copper

Time of exposure (h)	Mean copper concentration (μg/l)	Mean water hardness as $CaCO_3$ (mg/l)	Fraction dying	Fraction positive *Y. ruckeri*	Percentage mortality
12 (Cu+bacterium)	11·2±1·2	64·3±2·9	5/25	5/5	20
12 (bacterium only)	—	64·3±2·9	4/25	4/4	16
24 (Cu+bacterium)	11·2±1·2	64·3±2·9	9/25	9/9	36*
24 (bacterium only)	—	64·3±2·9	5/25	5/5	20
48 (Cu+bacterium)	11·2±1·2	64·3±2·9	11/25	11/11	44*
48 (bacterium only)	—	64·3±2·9	5/25	5/5	20
96 (Cu+bacterium)	11·2±1·2	64·3±2·9	9/25	9/9	36*
96 (bacterium only)	—	64·3±2·9	3/25	3/3	12
96 (control copper only)	11·2	64·3±2·9	0/25	0/0	—

*Significant at $\alpha = 0{\cdot}05$, by *t*-test.

longer periods were found to be no more susceptible to infection than those exposed to the bacterium only. No mortalities occurred in that group of fish exposed to copper only during the exposure time (16 days) and after being removed from the copper.

Effect of copper on LD_{50} of Y. ruckeri

The increase in infection susceptibility can be seen further in the decrease of the LD_{50} of *Y. ruckeri* for infection in those fish exposed to copper (Fig. 1). The LD_{50} for

Table 3. Effect of long-term, low-level copper exposure on *Yersinia ruckeri* infection susceptibility of steelhead trout

Time of exposure (days)	Mean copper concentration (μg/l)	Mean water hardness as $CaCO_3$ (mg/l)	Fraction dying	Fraction positive *Y. ruckeri*	Percentage mortality
1	5·7	44·0	8/25	8/8	32*
2	5·5	40·5	3/25	3/3	12
4	5·5	36·8	3/25	3/3	12
8	6·8	36·1	2/25	2/2	8
16	5·2	36·5	4/25	4/4	16
16	(control-bacterium only)	36·5	4/25	4/4	16
16	5·2 (control-copper only)	36·5	0/25	0/0	0

*Significant at $\alpha = 0{\cdot}05$, by *t*-test.

Y. ruckeri in copper-exposed fish was found to be $1{\cdot}9 \times 10^5$ bacteria per ml and $1{\cdot}1 \times 10^6$ in those fish exposed to the bacterium only. More fish died of the infection at each bacterial dosage in the copper-treated groups than in the control groups.

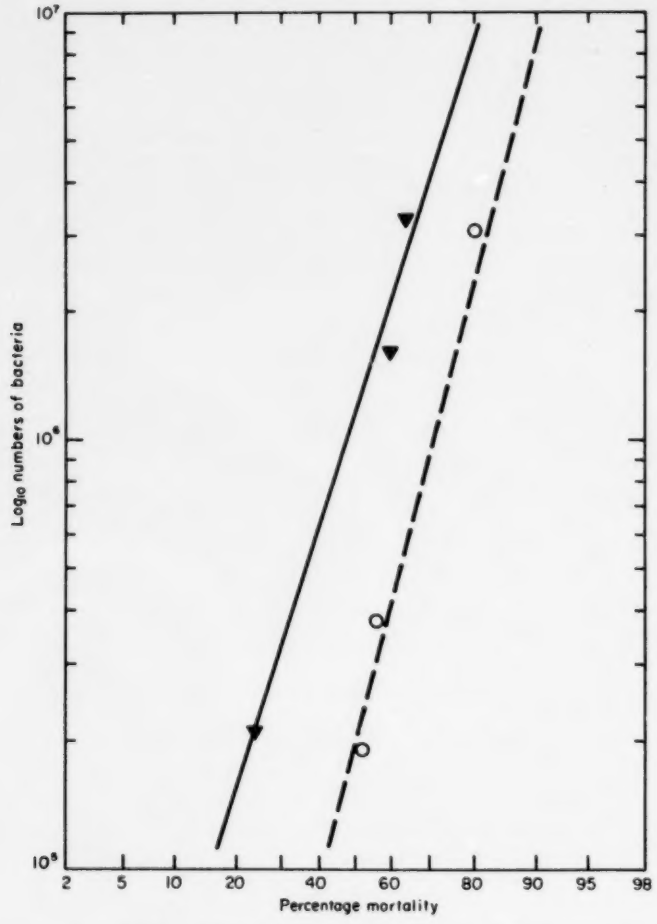

Figure 1. Log-probit plot of LD_{50} of *Yersinia ruckeri* in control (▲-▲) and copper (○-○) exposed fish. Fish exposed to mean copper concentration of $11{\cdot}7 \pm 1{\cdot}3$ μg/l for 48 h. Mean water hardness 22·3 mg/l as $CaCO_3$.

Discussion

The results presented here show the effect of sublethal copper exposure can be measured as increased susceptibility of the fish to infection. The copper stress is time-dependent as well as copper concentration-dependent. On examination of the data it

is found that those fish exposed for 96 h to copper concentrations of 7·0 and 10·6 μg/l were stressed (more susceptible to infection; Table 1) and fish exposed to copper below 7 μg/l were not affected. However, when fish were exposed to 5·7 μg/l (Table 3) only those fish exposed for 24 h exhibited any increased infection susceptibility. Thus, a copper concentration below 7 μg/l was found to be an effective stressor. The infection susceptibility would appear to be dependent upon the concentration of copper, as well as the time the fish are exposed to the copper. The lower the concentration (to a limit) the sooner the fish becomes susceptible and also the sooner it loses its susceptibility. At higher copper concentration, i.e., 10 μg/l, the longer it takes for susceptibility to occur and the longer it takes to lose or acclimatize to the copper stress. This point is supported by the data in Table 2; here the fish were exposed to 10 μg/l copper for up to 96 h. Those fish exposed for 24 h showed an increased infection susceptibility. This susceptibility increased after 48 h of copper exposure, but by 96 h of copper exposure the fish began to lose the susceptibility because similar mortalities occurred in this group as in those exposed for 24 h.

The toxicity of copper to fish and, in particular, steelhead trout has been well documented (e.g., Chapman 1978). The consequence of exposure of fish to sublethal doses of copper has been shown to elevate serum cortisol levels (Schreck & Lorz 1978). These authors also found that copper exposure lessened the survival of fish subjected to secondary stress such as handling or confinement. The results presented here show that another consequence of subacute copper exposure is an increase in disease susceptibility. The fishes' survival of sublethal toxicant exposure may not mean further survival if the fish are then exposed to a pathogen. Fish culturists have recognized that epizootics are often precipitated by stress, though this observation has little quantification. Certainly, a framework of experimentation has now been assembled that may allow further investigation into the effect of different kinds of stressors on disease outbreaks.

The overall effect of copper in reducing resistance to infection is not understood although one may speculate that the primary defence mechanisms such as white blood cell (WBC) counts may be decreased (McLeay 1975). A decrease of WBC would mean lower phagocytic activity and this would facilitate survival of any invading pathogen.

The present work is supported by the work of Hetrick *et al.* (1979) in that similar results were obtained. In both cases susceptibility is influenced by concentration of copper as well as time although this is less obvious in the work of Hetrick *et al.* (1979). The IHN virus LD_{50} was lowered in the presence of copper (Hetrick *et al.* 1979) as was the bacterial LD_{50} in the present work.

The present work with copper should be extended to include a survey of other toxic compounds to determine if the effects of sublethal intoxification can be measured by increased infection susceptibility. If it is found that infection susceptibility is a general phenomenon, it may be necessary to microbiologically examine mortalities occurring in toxicological tests to determine if the fish died of the toxicant alone or a combination of toxicant and pathogen.

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